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Plastid DNA variation in *Prunus serotina* var. *serotina* (Rosaceae), a North American tree invading Europe

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Abstract Black cherry (*Prunus serotina*) is a tree from North America, where it is often used for economical purposes, whereas it is widespread and invasive in Europe. Plastid DNA variation was first investigated in both its native and invasive ranges using microsatellite loci and sequences of three intergenic spacers (*trnT-trnL*, *trnD-trnT* and *trnS-trnG*). This analysis was focused on *P. serotina* var. *serotina*, with the inclusion of samples of closely related taxa. Length variation at a microsatellite locus (ccmp5) and a few sequence polymorphisms were identified among *P. serotina* samples. Four new primer pairs were then designed to specifically amplify variable regions and a combination of five markers was finally proposed for phylogeographic studies in *P. serotina*. These loci allow identification of six chlorotypes in *P. serotina* var. *serotina*, which may be particularly useful to depict the maternal origins of European invasive populations.

Keywords Microsatellite · PCR-RFLP · Plastid DNA · Population genetics

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Introduction

Black cherry, *Prunus serotina* Ehrh. (Rosaceae), is a deciduous tree native to an area ranging from Mexico-Guatemala to south-east Canada (McVaugh 1951; Little 1971). Based on phenotypic characteristics such as the height of the trees and the thickness of the leaves, five varieties are usually recognized: (1) *P. serotina* var. *eximia* (Small) Little is confined to the Edwards Plateau in central Texas; (2, 3) *P. serotina* var. *rufula* (Woot. & Standl.) McVaugh and *P. serotina* var. *virens* (Woot. & Standl.) McVaugh are distributed in Texas, New Mexico and Arizona; (4) *P. serotina* var. *salicifolia* (Kunth) Koehne is found from Mexico to Guatemala; and (5) *P. serotina* var. *serotina* which is the most abundant and common variety and is particularly widespread in the eastern part of the USA and Canada. Black cherry is one of the most valued cabinet and furniture woods in North America (Little 1971) but large, high-quality trees suited for commercial use (belonging to var. *serotina*) are found in a restricted range on the Allegheny Plateau of Pennsylvania, New York and West Virginia (Hough 1965; Marquis 1975).

Black cherry is now naturalized in several countries in northern South America, such as Ecuador and Peru (EPPO 2007). In Europe, *P. serotina* var. *serotina* was first introduced in the early seventeenth century. This tree was recorded in France near Paris around 1630 (Starfinger 2006), in England in 1629 (EPPO 2007) and in Germany in 1685 (Starfinger and Kowarik 2003), where it was particularly appreciated as an ornamental. Since the nineteenth century, the species was massively planted throughout Europe for timber production, but this objective was never achieved because the wood was of poor quality in the introduced range. The species is now widespread and invasive in north-eastern France, Belgium, the Netherlands, Germany, Denmark and Poland and locally abundant in

Lithuania, Romania, Hungary, Switzerland and Italy (EPPO 2007). Thanks to its tolerance to a very wide range of moisture conditions (Starfinger 2006), black cherry can invade open habitats such as dry grasslands and heathlands. *P. serotina* also benefits from anthropogenic influences such as soil disturbance and/or eutrophication (Godefroid et al. 2005). *P. serotina* disperses by seed (mainly through birds and frugivore mammals; Starfinger 2006) but also exhibits very efficient vegetative reproduction by suckering and sprouting. If sufficiently exposed to light, trees produce abundant quantities of seeds (up to 10,000 per tree in invasive populations; Pairon et al. 2006) after the age of approximately seven.

The use of genetic markers should be helpful to discern high-quality tree lineages in the native range and to depict the pattern of introduction of *P. serotina* in Europe (e.g. Freeman et al. 2007). The plastid genome (cpDNA) is usually inherited from the female parent (as already reported in other *Prunus* species; Brettin et al. 2000; Mohanty et al. 2003) and only dispersed by seeds. Thus, the use of cpDNA markers is often more efficient to detect colonization routes than nuclear markers (e.g. Petit et al. 2005) and is informative to depict the pattern of invasion. The combined use of plastid and nuclear markers is also very informative for a comparative study of gene dispersal by seeds versus pollen (seed- or pollen-mediated gene flow). However, plastid DNA variation can be particularly low in forest trees (e.g. Magri et al. 2007; Besnard 2008; Vendramin et al. 2008). Moreover, one can expect that European *P. serotina* populations could be genetically depauperate due to bottleneck effects, putatively leading to difficulties in the detection of useful polymorphisms for population genetic studies in the invasive area. Nevertheless, the successful use of highly variable cpDNA microsatellites has been reported for numerous trees (e.g. Harbourne et al. 2005; Magri et al. 2007) and such a methodology should be tested on *P. serotina*.

In the present paper, we investigate cpDNA polymorphism in *P. serotina* var. *serotina* using seven universal cpDNA microsatellite loci (Weising and Gardner 1999) and sequences of three intergenic spacers. Based on sequence polymorphism, new primers for the characterization of polymorphic loci are developed to easily characterize large samples of trees. Lastly, a combination of loci for cpDNA genetic studies of *P. serotina* var. *serotina* is proposed to allow efficient characterization of cpDNA variation in black cherry populations.

Material and methods

Plant sampling and DNA extraction

Eleven populations of *P. serotina* var. *serotina* were sampled throughout its distribution in both the native and invasive ranges (Table 1). Five individuals per population were collected at least 15 m from each other. In addition, two

Table 1 Description of the *Prunus serotina* and *P. virginiana* populations analysed in the present study, including population code (Pop. code), geographic location, GPS coordinates, number of individuals characterized (*N*) and detected haplotypes

Taxon	Location	Pop. code	GPS coordinates		*N*	Haplotypes[a]
			Latitude	Longitude		
Prunus serotina						
var. *serotina*	Native range (USA)					
	Charleston, South Carolina	SC1	32.45°N	79.54°W	5	ss4, ss5
	Spruce Knob, Virginia	WV1	38.71°N	79.54°W	5	ss4
	Ashville, Ohio	OH3	39.67°N	82.93°W	5	ss1
	Omaha, Nebraska	NE2	41.25°N	96.00°W	5	ss1, ss2
	Ames, Iowa	IO1	41.35°N	92.30°W	5	ss1, ss2
	Buckaloons, Pennsylvania	PE2	41.80°N	79.25°W	5	ss1, ss3, ss4
	Denton Hill, Pennsylvania	PE7	41.77°N	77.82°W	5	ss1, ss4
	Invasive range (Europe)					
	Ticino, Italy	IT1	45.52°N	08.72°E	5	ss6
	Fontainebleau, France	FR1	48.38°N	02.75°E	5	ss3
	Shakelford, UK	UK1	51.18°N	00.65°W	5	ss4
	Aarhus, Denmark	DA3	56.18°N	09.65°E	5	ss1, ss4
var. *rufula*	Native range (USA)					
	Tanque Verde, Arizona	AZ1	32.37°N	110.69°W	2	sr1
P. virginiana	Native range (USA)					
	Chloride, New Mexico	NM1	33.35°N	107.89°W	1	v1
	Cloudcroft, New Mexico	NM2	32.96°N	105.75°W	1	v1

[a] see Tables 2 and 3 for haplotype definition

individuals of both *P. serotina* var. *rufula* and *P. virginiana* (a species closely related to *P. serotina*; Shaw and Small 2004) were also collected in Arizona and New Mexico, respectively (Table 1). A total of 59 individuals were characterized. Approximately 20 mg of dry leaf material was ground in a 2-ml Eppendorf tube using a Tissuelyzer (QIAGEN) and three tungsten balls in each tube. Genomic DNA was extracted using a DNA-mini-extraction Kit (QIAGEN) according to the manufacturer's protocol.

Plastid DNA characterization

In order to characterize maternally inherited polymorphisms, seven cpDNA microsatellite loci (ccmp2, ccmp3, ccmp4, ccmp5, ccmp6, ccmp7 and ccmp10; Weising and Gardner 1999) were first tested on all samples. Each PCR reaction (25 µl) contained 10 ng DNA template, 1 × reaction buffer, 0.2 mM dNTPs, 0.2 µmol of each primer (one labelled with a fluorochrome), and 0.75 U *Taq* DNA polymerase (GoTaq, Promega, Madison, WI, USA). Reaction mixtures were incubated in a thermocycler (T1; Biometra, Göttingen, Germany) for 3 min at 94°C, followed by 36 cycles of 45 s at 94°C, 45 s at 53°C, and 60 s at 72°C. The last cycle was followed by a 10-min extension at 72°C. Electrophoresis procedures were used as described by Basic and Besnard (2006).

Three non-coding plastid regions (*trnT-trnL*, *trnD-trnT* and *trnS-trnG*) were sequenced on one *P. virginiana* tree (NM2) and eight *P. serotina* individuals originating from distant populations in the native (AZ1-1, NE2-1, PE7-2, IO1-7, SC1-2) and invasive ranges (UK1-1, IT1-8, FR1-4). For the PCR reactions, we used either universal primers for *trnT-trnL* (Taberlet et al. 1991) and *trnD-trnT* (Demesure et al. 1995) or new specific primers for *trnS-trnG* (*trnS-trnG*-For: 5′GTGAAACTTTGGTTTCATC3′ and *trnS-trnG*-Rev: 5′GAAAAAAGAGAAGACTATGTTAC3′). Each PCR reaction (50 µl) contained 10 ng DNA template, 1 × reaction buffer, 0.2 mM dNTPs, 0.2 µmol of each primer, and 0.75 U *Taq* DNA polymerase (GoTaq). Reaction mixtures were incubated in a thermocycler (T1; Biometra) for 3 min at 94°C, followed by 36 cycles of 45 s at 94°C, 45 s at 53°C, and 120 s at 72°C. The last cycle was followed by a 10-min extension at 72°C. PCR products were then purified using a QIAquick Purification Kit (QIAGEN) following the manufacturers' protocol. Direct sequencing was performed using Big Dye 3.1 Terminator sequencing cycle (Applied Biosystems) according to manufacturer's instructions and an ABI PRISM 3100 genetic analyser (Applied Biosystems).

Based on cpDNA sequences, four markers were developed to characterize our plant samples (Fig. 1). Primers (Table 2) were designed in regions flanking polymorphisms (i.e. nucleotide substitutions or length polymorphisms).

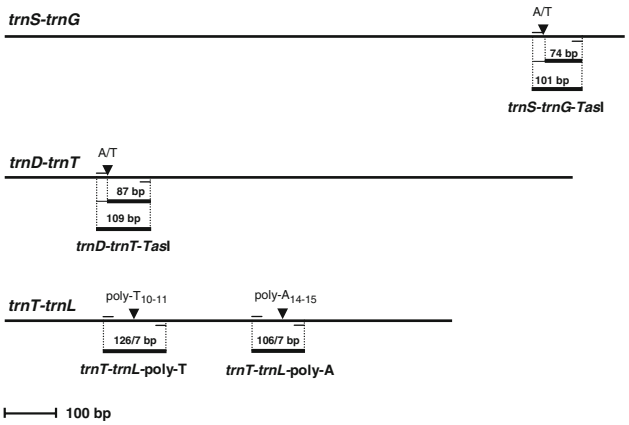

Fig. 1 Position of the two A/T nucleotide substitutions (A/T) and two length polymorphisms (poly-T or poly-A) detected in the plastid spacers *trnS-trnG* (1,225 bp), *trnD-trnT* (1,122 bp) and *trnT-trnL* (884 bp) of *Prunus serotina*. The four developed markers, with the position of each primer (–), are also indicated. For PCR-RFLP loci (*trnS-trnG-Tas*I and *trnD-trnT-Tas*I), the size of length variants is given after restriction by *Tas*I

Each PCR reaction (25 µl) contained 10 ng DNA template, 1 × reaction buffer, 0.2 mM dNTPs, 0.2 µmol of each primer (one labelled with a fluorochrome; Table 2), and 0.75 U *Taq* DNA polymerase (GoTaq). Reaction mixtures were incubated in a thermocycler (T1; Biometra) for 3 min at 94°C, followed by 36 cycles of 30 s at 94°C, 30 s at the defined annealing temperature (Table 2) and 30 s at 72°C. The last cycle was followed by a 10-min extension at 72°C. Furthermore, in *trnD-trnT-Tas*I and *trnS-trnG-Tas*I, two T/A nucleotide substitutions (leading to *Tas*I restriction-site polymorphisms) were investigated using the restriction fragment length polymorphism (RFLP) methodology. Digestion of the PCR fragments was performed using the restriction enzyme *Tas*I (Fermentas, St Leon-Rot, Germany) according to the manufacturer's recommendations. Electrophoresis procedures were used as described by Basic and Besnard (2006).

Data analysis

The total gene diversity (H_T) was estimated using F_{STAT} (Goudet 2005) for both native and invasive populations of *P. serotina* var. *serotina*. This parameter was estimated for each locus and on the multi-locus profiles (considered as alleles from one locus). The mean within-population gene diversity (H_S) was also estimated for native and invasive populations on the multi-locus profiles. A test for a significant difference of within-population gene diversity between native and invasive populations was ascertained using comparisons among groups of samples as implemented in F_{STAT} with 10,000 permutations of individuals among groups tested. In addition, a reduced median network for *P. serotina* haplotypes was built based on the combined PCR-RFLP

Table 2 Characteristics of new primers used to characterize cpDNA polymorphisms in *Prunus serotina*, including annealing temperature (T_a) in °C, the number of alleles (N) and the size of alleles in bp

Locus	GenBank	Primers	T_a	N	Allele sizes[a]
*trnS-trnG-Tas*I	AM950170 to AM950177	For:5′CTATAATCATAGAAATCTAAATAAACA3′	47	2	A) 101 → 74[b] + 27
		Rev: FAM-5′TCGTAAATAAACTGATTTATTTGATT3′			B) 101 → 101[b]
*trnD-trnT-Tas*I	AM950153 to AM950160	For: 5′GGATAATCACTCTTTCAATGT3′	50	2	A) 109 → 87[b] + 22
		Rev: HEX-5′AATTCTGATCTTGCTAATGATC3′			B) 109 → 109[b]
trnT-trnL-poly-T	AM950162 to AM950169	For: FAM-5′ATTAGCTTAATTAGATAGTAAG3′	47	2	126, 127
		Rev: 5′CCGTTAATTTATAATTAGAAGA3′			
trnT-trnL-poly-A	AM950162 to AM950169	For: 5′TAATTCAGATCATAATGAAACA3′	47	2	106, 107
		Rev: FAM-5′GTGCAATTTTGAATACTTGAA3′			

[a] For *trnS-trnG-Tas*I and *trnD-trnT-Tas*I, the size of fragments before and after (→) restriction is given

[b] DNA fragment labelled with the fluorochrome (FAM or HEX)

Table 3 Multi-locus profiles of the eight cpDNA haplotypes detected in *Prunus serotina* (ss1 to ss6 in var. *serotina*, and sr1 in var. *rufula*) and *P. virginiana* (v1)

Locus	Multi-locus profile (haplotype)								$H_{T(N)}$	$H_{T(I)}$
	ss1	ss2	ss3	ss4	ss5	ss6	sr1	v1		
ccmp2	204	204	204	204	204	204	204	202	0	0
ccmp3	105	105	105	105	105	105	105	125	0	0
ccmp4	118	118	118	118	118	118	118	118	0	0
ccmp5	126	126	126	126	125	126	129	127	0.057	0
ccmp6	111	111	111	111	111	111	111	110	0	0
ccmp7	133	133	133	133	133	133	133	133	0	0
ccmp10	113	113	113	113	113	113	113	113	0	0
*trnS-trnG-Tas*I	101	101	74	101	101	101	101	68	0.057	0.375
*trnD-trnT-Tas*I	87	87	87	109	109	109	87	86	0.514	0.488
trnT-trnL-poly-T	126	126	126	126	126	127	126	131	0	0.375
trnT-trnL-poly-A	106	107	106	106	106	106	106	107	0.248	0
Total	–	–	–	–	–	–	–	–	0.661	0.738

The length (in bp) of each sized DNA fragment is given. Gene diversities in the native [$H_{T(N)}$] and invasive [$H_{T(I)}$] ranges are given for the *P. serotina* var. *serotina* populations

and microsatellite data. Nucleotide polymorphisms were coded as present/absent and length variations at microsatellite loci were coded as multistate characters. This maximum-parsimony analysis was performed using the NETWORK software (http://www.fluxus-engineering.com/sharenet.htm; Bandelt et al. 1999).

Results and discussion

The characterization of all samples with seven ccmp loci (Weising and Gardner 1999) allowed clear distinction between *P. virginiana* and *P. serotina* (using length variation at ccmp2, ccmp3, ccmp5 and ccmp6; Table 3). This result supports that these two species are phylogenetically differentiated based on maternal markers. In addition, our samples of *P. serotina* var. *serotina* and var. *rufula* can be distinguished based on ccmp5 (Table 3). In *P. serotina* var. *serotina*, only one locus (ccmp5) was variable with one tree from SC1 (SC1-2) displaying a rare variant (i.e. 125 bp).

The lack of genetic polymorphism in *P. serotina* var. *serotina* revealed by the ccmp loci led us to investigate DNA sequence polymorphism in the intergenic spacers *trnT-trnL* (884 bp), *trnD-trnT* (1,122 bp) and *trnS-trnG* (1,225 bp). In *P. serotina*, only four variable sites were found (Fig. 1), but no new specific polymorphism was detected in the sample of var. *rufula* compared to samples of var. *serotina*. On the *trnD-trnT* and *trnS-trnG* spacers, one T/A substitution (creating *Tas*I restriction-site polymorphism) was found at positions 200 and 1061, respectively. On *trnT-trnL*, two length polymorphisms of 1 bp were found at positions 257 and 551, respectively, in a poly-T ($T_{10–11}$) and a poly-A ($A_{14–15}$).

Four cpDNA primer pairs were developed (Table 2; Fig. 1) to amplify and characterize these polymorphisms on all samples. Length variation revealed on both *trnT-trnL* markers (poly-T and poly-A) was fully congruent with our

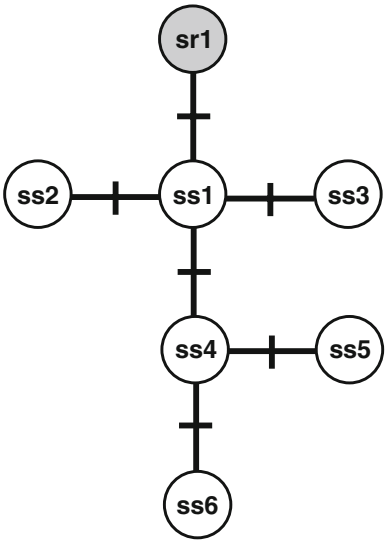

Fig. 2 Reduced median network representing phylogenetic relationships of plastid DNA haplotypes of *Prunus serotina* var. *serotina*. Each haplotype is coded as reported in Table 3. Haplotype sr1 (*P. serotina* var. *rufula*) is used as outgroup. This analysis is performed on five polymorphic loci

sequence data. Our markers revealed various levels of gene diversity (H_T) and the most discriminating locus in both ranges was *trnD-trnT-Tas*I ($H_T \approx 0.5$; Table 3). Combining these loci with ccmp5, six haplotypes (named ss1, ss2, ss3, ss4, ss5 and ss6) were found in *P. serotina* var. *serotina* (Table 3). The reconstructed network (Fig. 2) showed that haplotypes are all interconnected with ss1 and ss4 at central positions. The outgroup haplotype (sr1) is branched on ss1 suggesting that the latter probably represents the ancestral profile. Three haplotypes (ss1, ss3 and ss4) were shared by both native and invasive populations, two haplotypes (ss2 and ss5) were just detected in the native area and one haplotype (ss6) was only found in the invasive range. Surprisingly, in our sample the global gene diversity was similar between the native ($H_T = 0.661$) and invasive ranges ($H_T = 0.738$). The mean within-population gene diversity was also not significantly different between native ($H_S = 0.329$) and invasive ($H_S = 0.150$) populations ($P = 0.241$). These observations suggest that there was not important reduction of gene diversity during the invasion. Interestingly, intrapopulation variation was also detected in several populations and even in the invasive range (PE2, PE7, IO1, NE2, SC1 and DA3; Table 1).

This study has shown that, even in the presence of low sequence polymorphism, substantial cpDNA diversity can be detected throughout the *P. serotina* distribution. Plastid DNA markers may be very useful for population genetic studies in *P. serotina*, and particularly here for var. *serotina* for which we recommend the combined use of three microsatellite loci (i.e. ccmp5, *trnT-trnL*-poly-T and *trnT-trnL*-poly-A) and two PCR-RFLPs (i.e. *trnS-trnG-Tas*I, *trnD-trnT-Tas*I). Moreover, their combined use with nuclear SSR markers (Pairon et al. 2008) will also help to depict the origins and dispersal of invasive populations in Europe. The presence of distinct cpDNA haplotypes in Europe supports the hypothesis of multiple introductions in the invasive range. The molecular characterization of a more exhaustive sample in both the native and invasive ranges is now necessary to better document these introductions and then identify the pattern of colonization of this invasive species (M. Pairon in prep.; B. Petitpierre et al., in prep.). Detection of the different invasive forms could also open new perspectives to understand invasion of the tree, particularly by integrating such information in niche-based modelling (Mau-Crimmins et al. 2006; Broennimann et al. 2007).

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